Biophysical Approaches to Assess the Stability of Allergens

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1. Introduction

Stability of the protein structure is considered an important characteristic of food allergens. Proteins stable toward digestion in the gastro-intestinal tract supposedly reach the gut immune system rather intact, and may give rise to allergic sensitization. Furthermore, proteins with high stability are less susceptible for thermal denaturation, such that heat treatment (food processing) is not suitable for decreasing protein allergenicity. The issues of protein digestibility and the effects of food processing on the protein structure will be discussed elsewhere in this publication, by Drs. Murch and Wickham, and Dr. Pascke, respectively. The objective of this chapter is to discuss the biophysical approaches to assess the stability of protein structures. Two distinct issues will be discussed: 1) protein stability at elevated temperature, related to food processing, and 2) protein stability at ambient temperature, related to digestability. Techniques that are in place to investigate the protein's structural stability will be discussed using two examples. Ara h 1, a major peanut allergen from peanut will be used as a model for heat-induced structural changes, and Ber e 1 form Brazil nut will be used to illustrate the implications of protein structure for digestibility.

2. Overview of techniques for assessing the stability of a protein structure

The structure of a protein is classically described at four levels: primary, secondary, tertiary, and quaternary structure.

Primary structure

The primary structure of a protein is the amino acid sequence, as encoded for each protein by the genetic material. For many proteins and allergens these sequences have been reported [1,2]. Edman degradation can be used to determine amino acid sequences. With this method, the N-terminal amino acid is removed form the protein, and identified using chromatography. A disadvantage is that Edman degradation is restricted to peptides/proteins of limited size. Per run, maximally about 30-50 cycles can be done, resulting in a partial sequence of 30-50 amino acids. Larger peptides should be predigested, and the resulting peptides purified before Edman analysis. This makes it time consuming, and expensive. Alternatively the primary sequence can be derived from analysis of the genetic code once this has been identified.

The use mass-spectrometry (MS) is an alternative for Edman degradation analysis. This method makes amino acid sequencing more easy and rapid, especially with automated liquid chromatography (LC) as separation step prior to MS. In-gel protein digestion followed by LC-MS gives information about the peptide masses, making isolation of the proteins or derived fragments redundant and allows the sequencing of complex mixtures of proteins. A consecutive MS step enables to identify the peptides, and the combined information may lead to a complete amino acid sequence. This approach, LC- MS/MS, is often used in proteomics.

The primary structure holds all information for all features of a protein, including the folding trajectory and the final and unique three-dimensional structure. However, by knowing the primary structure alone, no predictions can be made *ab initio* about a protein's structural stability. Newly identified proteins with an amino acid sequence homologous to proteins that are further characterized (structure analysis), will probably resemble a similar structure and can be classified in certain families that bear resemblances in structure and to some extent in stability. Although the generality of a prediction is limited, insight in the primary sequence is a first step in considering protein stability.

Secondary structure

With the secondary structure of a protein the local arrangement of a protein chain into defined structures (α-helices, β-sheets, and β-turns) is meant. Hydrogen bonds between the C=O

(carbonyl) and N-H (amide) groups of the peptide bond between individual amino acids stabilize the secondary structure. There are two methods most frequently employed to determine the relative amount of secondary structure elements, far-UV circular dichroism (far-UV CD) and Fourier-transform infra red spectroscopy (FT-IR), with far-UV CD being the most popular.

Far-U CD is based on the chirality of the nitrogen atom in an individual peptide bond that links two amino acids. This chirality results in a more pronounced absorption of left or right polarized far-UV radiation, which is measured as ellipticity, usually expressed as milli degrees (mdeg). A far-UV CD spectrum is normally recorded from approximately 190 nm to 240 nm, and α-helices, β-structures have their own typical spectra (Table 1). Regression analysis of a recorded spectrum with reference spectra of α-helices, β-sheets, and β-turns gives an estimation of the percentage of the secondary structure elements relative to un-organized amino acid stretches (random coil). Proteins need to be soluble for this analysis with far-UV CD spectroscopy, and typically a concentration of 0.1 mg/ml is used in a neutral buffer with extreme low absorbing in the UV wavelengths (phosphate). Far-UV CD spectroscopy can be done at elevated temperatures (up to 80-90 ˚C), to investigate heat-induced unfolding.

The infra red (IR) absorbance of the amide (N-H) group is indicative of the secondary structure. The spectral region where the typical amide I band adsorbs is form 1700 cm^{-1} to 1600 cm^{-1}. Wave numbers of absorbance maxima can be used to assign α-helices and β-structures in a qualitative way (Table 1). It is not necessary to have soluble protein because of the large wave length of the IR radiation.

***Table 1**. Some characteristics of secondary structure elements*

	Far-UV CD		**FT-IR**
α-helices	Positive extreme at 197 nm and negative extremes at 208 and 222	Zero crossing[1)] at 200.8	Absorption maximum around 1655 cm^{-1}
β-strand/ β-sheet	Positive extreme at 198 and negative extreme at 217 nm	Zero crossing[1)] at 207	Absorption maximum around 1685 and 1635 cm^{-1}

[1)] At these wavelengths, the net ellipticity is 0, so that at these wavelengths the observed ellipticity originates from other secondary structure elements. These values can thus be employed for one-wave length screening at variable temperature to monitor changes in defined secondary structure elements.

Tertiary structure

The tertiary structure of a protein is the spatial orientation of secondary structure elements (α-helices, β-structures). Most often the tertiary packing of a protein is referred to when one speaks about the globularity of a protein. Forces that contribute to tertiary folding include: hydrogen bonds, hydrophobic bonds, ionic bonds, and disulphide bonds (-S-S- bonds). The secondary structure elements can be held together tightly, or be more loosely associated. When tightly packed, the exposure to water is less compared to the situation where the protein adopts a loose packing. The aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) have fluorescent properties that depend on the polarity of their environment. In practice only the fluorescence yield of Trp is sufficiently high for performing fluorescence spectroscopy. Changes in hydrophobicity of the environment of the aromatic amino acids will result in shifts of the fluorescence maxima and the intensity of the emitted light. Thus heat-induced shifts in fluorescent maxima or changes in intensity can be used to monitor protein unfolding (towards more water-exposed) or aggregation (towards more buried in the hydrophobic core of the protein).

Similar to far-UV CD, near-UV CD is a spectroscopic tool that uses chirality in proteins, but in this case of an asymmetric arrangement of π-electron clouds of aromatic groups. If such aromatic group is enclosed in a defined 3D-structure it becomes likely that both sides of an aromatic ring face different local environments. This asymmetry results in a chirality that diminishes when

such ring regains its rotational freedom for example as a result of unfolding of the 3D-structure. Aromatic amino acids absorb in the near-UV spectral range (260 -350 nm). Phe absorbs typically in the 260-290 nm range, Tyr in the 280-300 nm, and Trp in the 300-325 nm spectral range. Table 2 gives some characteristics of near-UV CD en tyrosine fluorescence spectroscopy.

Another approach to investigate the stability of the tertiary packing of a protein is monitoring the energy needed to increase the temperature of a system containing protein. Upon unfolding, the energy consumption for temperature increase is higher compared to buffer alone. This difference is indicative for endothermic transitions. This technique is referred to a differential scanning calorimetry (DCS) and can be used for concentrated protein solution. A disadvantage is that relatively high amounts of protein are needed (1 ml with a concentration of 5 mg/ml). By cooling down the system in a controlled way, possible reversible transitions can be detected. The denaturation temperature, energy content of the transition (size, negative or positive), as well as the reversibility of the transition provide information about the protein denaturation, making DCS a powerful tool in investigating protein stability, even although a large amounts of protein is required. In some cases also exothermic transitions can be observed that are often related to extensive protein aggregation phenomena.

***Table 2**. Spectral characteristics of aromatic amino acids related to tertiary structure*

	Near-UV CD spectroscopy Absorbance range (nm)	**Fluorescence spectroscopy** Emission maximum (nm)
Phenylalanine	260-290	Not applicable [1)]
Tyrosine	280-300	Approximately 310 [2)]
Tryptophan	300-320	Water exposed: 353
		Burried in protein core: 330-335

[1)] Quantum yield very low

[2)] The emitted light of a tyrosine is in most cases absorbed by tryptophans and therefore difficult to detect.

Quaternary structure

With the quaternary structure of a protein the association of individual proteins is meant. Often the arrangement of these proteins is well defined. In some cases one also talks about subunits in a

quaternary structure. In these cases a single folded protein is proteolytically cleaved yielding two strands of amino acids not linked anymore via a peptide bond, while in some cases other covalent links are present like S-S bonds. Single chain proteins have therefore, by definition, no quaternary structure. Oligomers of identical subunits (tri-meric Ara h 1), and hetero-multimers composed of different subunits (heavy chain associated to light chain of Ber e 1) are examples of quaternary structures. The subunits themselves may have intrinsic tertiary and secondary structure and stability. Differences in the apparent molecular weight of a protein between different conditions (high versus low ionic strength, reducing versus oxidative, with or without detergent) are indicators for quaternary structure. This can be visualized by means of SDS-PAGE analysis, ultra-centrifuge experiment in sucrose gradients (UC), gel permeation chromatography (GPC), all with their own characteristics. A combination of these methods is needed to obtain a full picture of the quaternary structure of a protein.

Table 3. *Some characteristics of methods to elucidate a protein's quaternary structure*

	Units	**Disulphide bonds**	**Other features**
SDS-PAGE	kDa	Reduced: individual chains; non-reduced: one complex	SDS dissociates non-covalent interactions
Ultracentrifugation	S (Svedberg sedimentation coefficient) [1)]	Normally non-reduced, reduced possible when alkylated	Time consuming
Gel permeation chromatography	S (Svedberg sedimentation coefficient) [1)]		Effect of ionic strength can be sorted out

[1)] For globular proteins, ultracentrifugation and gel permeation chromatography can be calibrated by means of (globular) proteins with known size.

Protein structure and allergenicity

To trigger allergic responses to proteins recognition of protein specific characteristics is required. This recognition can be at different levels. Some specific primary amino acids sequences can act as epitope. But some cases have been reported where the allergenicity is related to the formation of a defined secondary structure that brings a number of specific amino acids in a very constrained distance of each other. In other cases that proximity was not related to a secondary

fold, but to a tertiary structure, allowing amino acids that are far apart in the primary sequence to be close in space allowing them to serve as template for epitope recognition. Quaternary structure can generally be regarded as a hampering effect for epitope recognition, since parts of proteins can be shielded from the solvent. Clearly, a protein structure, and even more important the stability of the structure is a major determinant in understanding and controlling allergenicity. In the following two examples the impact of destabilization of the structure of two allergens is discussed.

Example 1: Investigation of heat-induced structural changes of Ara h 1

Based on: Koppelman et al [3] and Shin et al [4].

Background

Ara h 1 was purified from both raw and roasted peanuts to homogeneity (>95% purity). Ara h 1 from raw peanuts was characterized on secondary, tertiary and quaternary folding level, and subsequently heated to investigate conformational changes. The results were compared with Ara h 1 obtained from roasted peanuts.

Temperature effects on secondary folding level of Ara h 1

Figure 1 shows the far-UV CD spectrum from Ara h 1 obtained from raw peanuts. Spectral analysis to obtain an estimation of the secondary structure content reveals 31% α-helices, 36% β-structures, and 33% random coil. These data are confirmed by FT-IR (Figure 2), where the amide I band shows a maximum at 1635 cm-1 (β-structures) with a clear shoulder around 1655cm-1 (α-helices).

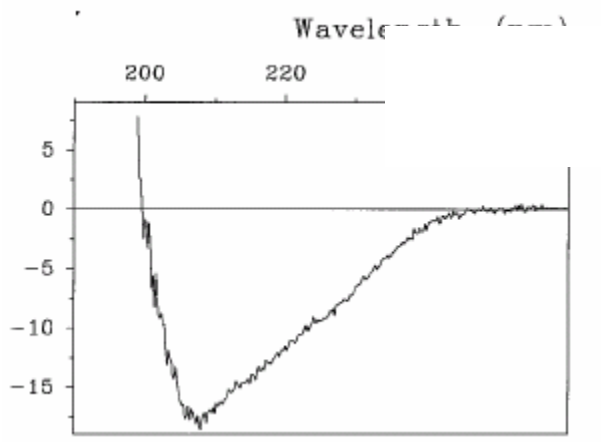

Figure 1. *Far UV-CD spectroscopy of Ara h 1 from raw peanuts at 20 °C*

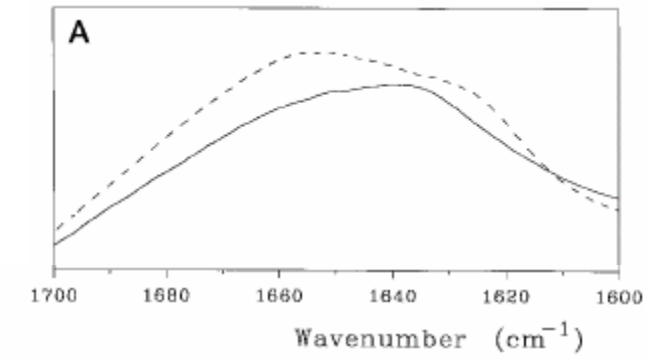

Figure 2. *FTIR spectra from Ara h 1 at 20 °C*
Solid line: before heating; dotted line after heating up to 90 °C and cooling down to 20 °C.

Increasing the temperature clearly affects the shape of the amide I band in FTIR (Figure 2), resulting in a more pronounced shoulder at 1630 cm^{-1} indicating the formation of (anti-parallel) β-structures. Also, an increased intensity at 1658 cm^{-1} is observed. Apparently, denaturation leads to a more a secondary more structured conformation of the protein. This could explain the observation that Ara h 1 aggregates upon heating, a process that is known to coincide with the formation of anti-parallel β-sheet structures. Because of the observed aggregation, far-UV CD spectroscopy is not possible at elevated temperature.

Temperature effects on tertiary structure of Ara h 1

In order to further explain the denaturation as described above, the tertiary structure of Ara h 1 was studied at ambient temperature, and at increasing temperatures. To assess the denaturation temperature, DSC was performed. A clear endothermic transition can be observed in Figure 3, with an onset temperature of 83 ˚C and a maximum of 87 ˚C. The energy content of this transition is 30 kcal/mol, which is low in comparison with other globular proteins. This might indicate that the denaturation is only partial rather than complete. This is in agreement with the far-UV CD data, showing an increase in secondary structure. Upon cooling of the sample no transition was observed, demonstrating that the unfolding was irreversible and complete, as indicated by the re-heating scan.

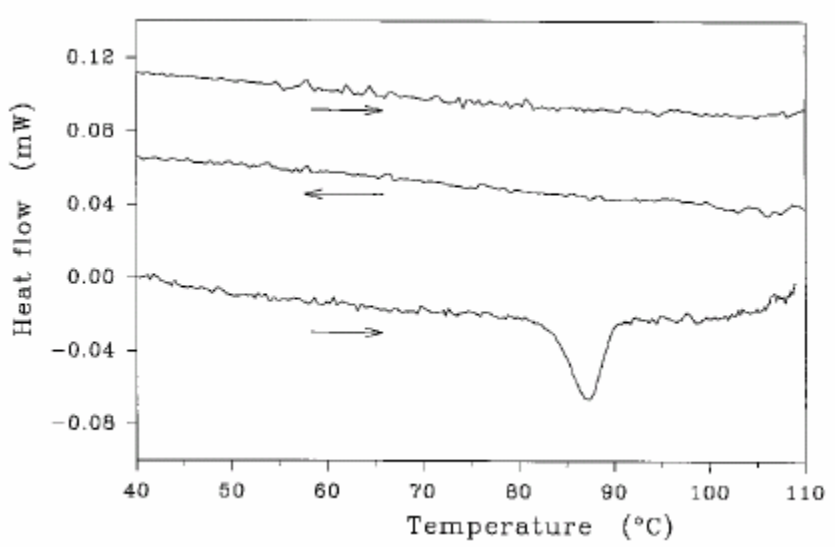

Figure 3. *Differntial scanning calorimetry of Ara h 1.*
The arrows indicate whether the trace is heating or cooling. Traces are vertically displaced to improve the clarity of the figure.

In order to further evaluate the denaturation at a tertiary folding level, near-UV CD spectra were recorded (Figure 4a) first at ambient temperature. This demonstrates a distinct tertiary fold similar as reported before for some other plant storage proteins. Tryptophan fluorescence data show an emission maximum at 348 nm, close to that of tryptophan that is water exposed (350 nm, Table 2). This indicates that the tryptophan residues in Ara h 1 are relatively on the outside of the protein, making this method less suitable for studying unfolding. Monitoring the

tryptophan fluorescence as a function of heating showed only a linear decrease in intensity, which is an intrinsic property of tryptophan fluorescence and does not reflect altered tertiary packing. Upon heating (Figure 4b), the CD intensity at 288 nm at 80˚C is only decreased 25%, while at 313, the reduction is 65%. This indicates that the tryptophan residues present gain upon heating more rotational mobility due to reduced local packing, than the phenylalanine residues. The domain where the majority of the tryptophan residues reside (N-terminal) has a low stability. The near-UV CD of the phenylalanine residues, however, is hardly affected by temperatures up to 80 ˚C, indicating these residues are located in the core of the Ara h 1 molecule. Indeed, as suggested by the comparison with the aligned structure of another plant storage protein, phaseolin, of which the 3D-structure has been resolved.

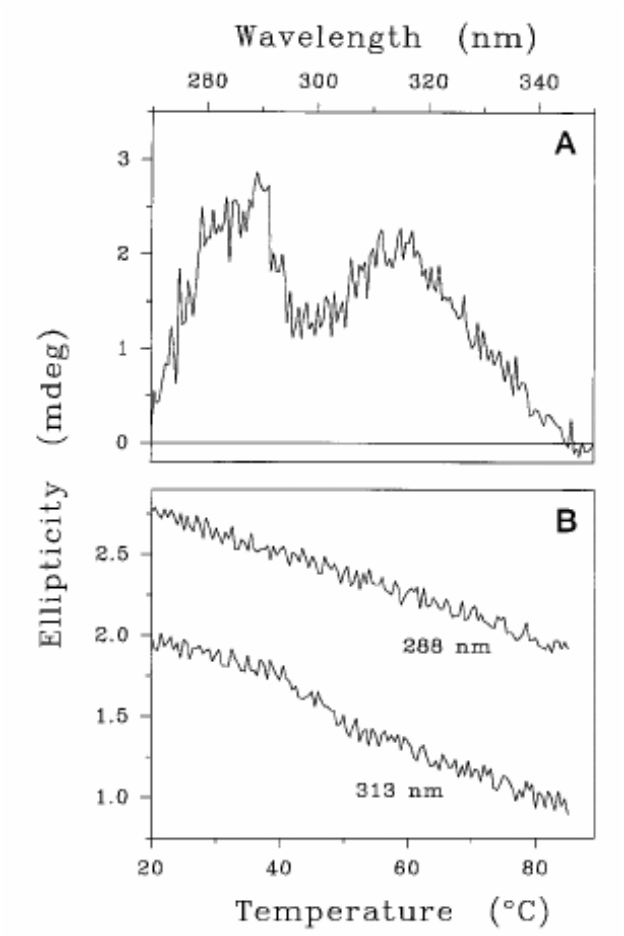

Figure 4. *Tryptophan fluorescence of Ara h 1*
Panel A: spectrum recorded at 20 °C. Panel B: fluorescence at 288 nm and 313 nm as a function of temperature.

Quaternary organization of Ara h 1

Tri-mers of Ara h 1 have been described by several investigators. GPC indicated a molecular weight of approximately 200 kDa, while on SDS-PAGE, both under reducing and non-reducing conditions a single band of approximately 63 kDa was observed. This SDS-PAGE pattern indicated that the SDS either in absence or presence of reducing agents could dissociate the interaction. So, hydrophobic interactions are thought to play an important role in holding the tri-mer together. Shin et al [4] studied this quaternary organization in more detail. At low concentrations (10 nM), Ara h 1 is a monomer, while at higher concentrations (50 nm and upwards) Ara h 1 adopts a tri-meric organization. This was shown by fluorescence anisotropy of fluorescein-labeled Ara h 1 (Figure 5) as a function of the concentration of added (unlabeled) Ara h 1. Association of monomeric fluorescein-labeled Ara h 1 with unlabeled Ara h 1 into tri-mers results is a decrease of rotational diffusion of the labeled species and consequently an increase in polarization (Figure 5, Y-axis). Increasing the temperature leads to aggregated Ara h 1 composed of monomers, tri-mers and higher order multimers [3]. This aggregation occurred at the denaturarion temperature of Ara h 1 and is concentration dependent (Figure 6) [3].

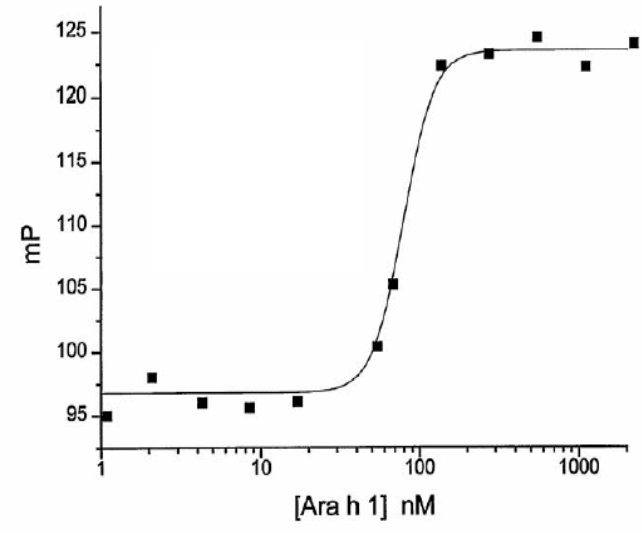

Figure 5. *Concentration-dependent formation of Ara h 1 tri-mers at ambient temperature [4]*
Y-axis: fluorescence anisotropy (polarization).

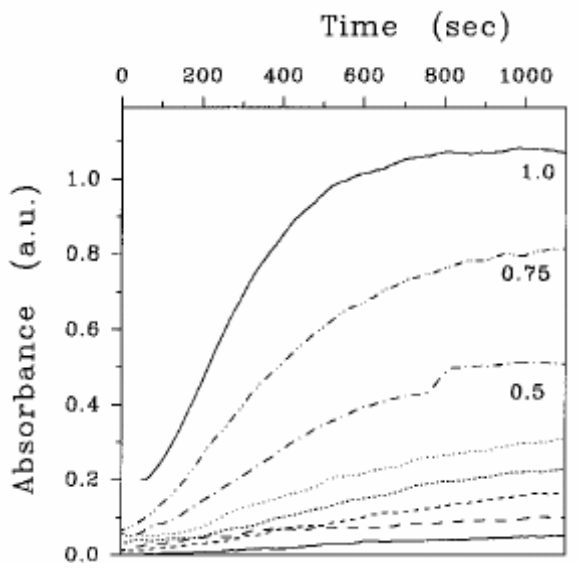

Figure 6. *Aggregate formation of Ara h 1 at its denaturation temperature (85 ˚C)*
Numbers indicate the Ara h 1 concentration in mg/ml.

Structure data obtained from Ara h 1 purified from roasted peanuts largely confirmed the heat-induced structural changes of Ara h 1 from raw peanuts [3].

Together, the data of the structural analysis of Ara h 1 at ambient ant elevated temperature show that heat treatment of Ara h 1 gives rise to an irreversible endothermic transition of Ara h 1 at 85 ˚C inducing an increase in β-structures, accompanied with aggregation of the protein.

Example 2: Investigation of structural aspects in relation to the digestion of Ber e 1
Based on: Koppelman et al [5]

Background
Ber e 1, a 2S albumin, is the major allergen from Brazil nut and is organized as a hetero-dimer consisting of a 9 kDa heavy chain and a 3 kDa light chain, both originating from the same gene and expressed as a single chain peptide that is proteolytically processed. The heavy and light chain are held together by 4 disulphide bonds. Ber e 1 was purified to homogeneity and the stability of its structure was characterized. The consequences of this structural stability for the digestibility by protease was investigated by generating two forms of Ber e 1: native Ber e 1 and

Ber e 1 in which the disulphide bonds were reduced and alkylated such that the heave chain was separated from the light chain. Figure 7 shows the native Ber e1 and reduced and alkylated Ber e 1 (RA-Ber e 1). The native Ber e 1 migrates at a lower molecular weight than expected, probably caused by a tight globular packing. Interestingly, reduction of the disulphide bonds using regular reducing SDS-PAGE sample buffer is not sufficient to separate the heavy and light chain of Ber e 1. It does, though, affect the globularity, as indicated by an increased apparent molecular weight (Figure 7, lane 2 compared to lane 1).

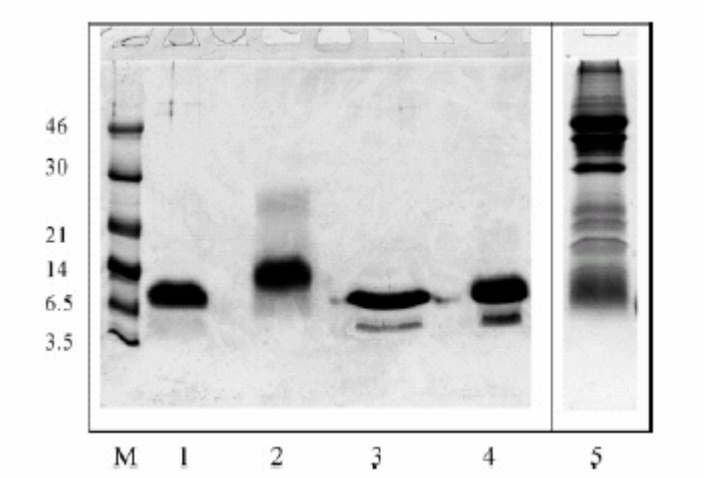

Figure 7. *Different preparations of Ber e 1*
Lane 1: native Ber e 1 analyzed under non-reducing conditions on SDS-PAGE; Lane 2: native Ber e 1 analyzed under reducing conditions on SDS-PAGE; Lane 3: RA-Ber e 1 analyzed under non-reducing conditions on SDS-PAGE; Lane 4: RA-Ber e 1 analyzed under reducing conditions on SDS-PAGE; Lane 5 Ber e 1 extract. M: Markers, indicated in the left margin (kDa).

For the highly conserved family of 2S proteins, a three dimensional model has been constructed based on a sequence- and structural alignment with napin seed storage protein. The tight globular packing is dominated by the presence of 4 disulphide bonds, and the disulphide bonding is thought to contribute to the stability to a large extent. Reduction of this bond was therefore believed to have a large impact on the digestability of the protein

Secondary structure of Ber e 1; Eeffect of heat treatment and unfolding at ambient temperature

The secondary structures of native Ber e 1 and RA-Ber e 1 were analyzed by far-UV CD spectroscopy, both at pH 7.0 and pH 2.0. The spectra (not shown) indicate a high degree of

secondary structure with an estimated α-helix content of 30% (both at pH 2.0 and pH 7.0) and 40% and 46% β-structures at pH 2.0 and pH 7.0, respectively. These figures were confirmed by IR spectroscopy (not shown) [5]. Interestingly, upon reduction and alkylation, the α-helix structures remained, while the β-structures were completely lost as analyzed by far-UV spectroscopy. IR spectroscopy confirmed the increase in random coil [5].

The effect of heat treatment on the secondary structure was investigated and compared with the effect of denaturation at ambient temperature by using urea-induced unfolding. Figure 8B shows the corresponding far-UV CD spectra.

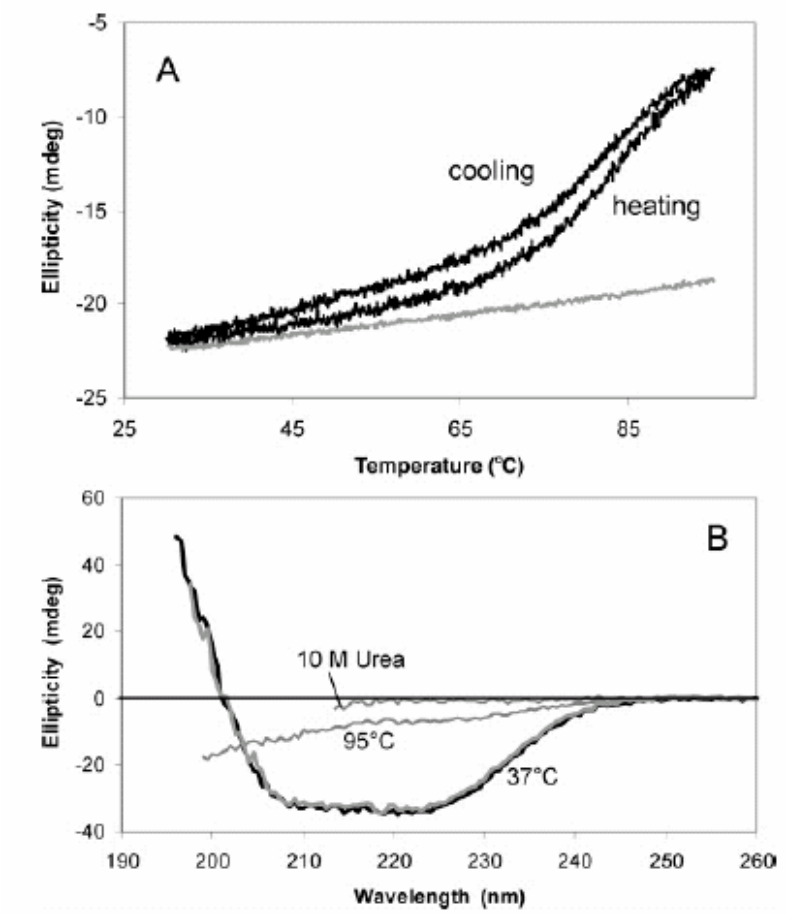

Figure 8. *Far-UV CD spectroscopy of Ber e 1.*
Panel A: ellipticity at 222 nm (indicative for both α-helices and β-structure) as a function of temperature. Gray line: at pH 7.0; black lines: at pH 2.0 (heating and cooling). Panel B: Spectra from native Ber e 1 before (black line) and after heat treatment at pH 7.0 (gray line) recorded at 37 °C. Denatured Ber e 1 (heat treated at pH 2.0, spectrum recorded at 95 °C) and urea-unfolded Ber e 1 are shown as comparison.

The secondary structure content of native Ber e 1 remains up to 90 ˚C when heated at pH 7.0, while at pH 2.0, a reversible denaturation occurs (Figure 8, panel A). At 95˚C, there is a strong, but not complete loss of secondary structure, as shown by the spectrum of Ber e 1 in 10 M urea, representing Ber e 1 in a completely non-structured way. These data show that at pH 2.0 Ber e 1 is more susceptible to denaturation compared to the situation at pH 7.0. On a tertiary folding level, DSC experiments showed that both native and RA-Ber e 1 at pH 7.0 did not undergo a transition (measured up to 110 ˚C), while at pH 2.0 native Ber e 1 denatured at 82.5 ˚C and RA-Ber e 1 at a similar temperature of 81.3 ˚C. However the energy content of these transitions were rather different (320 kJ/mol for native Ber e 1 and 56 kJ/mol for RA-Ber e 1). These data are in line with the decreased secondary structure content Ber e 1 upon reduction and alkylation.

This observed decreased stability was further explored by guanidinium titration, where adding the chaotropic salt guanidinium shifts the balance between folded and unfolded conformers (figure 9). This approach allows obtaining information of the stability of the protein structure at any desired temperature (i.e. 37 ˚C), in contrast to DCS.

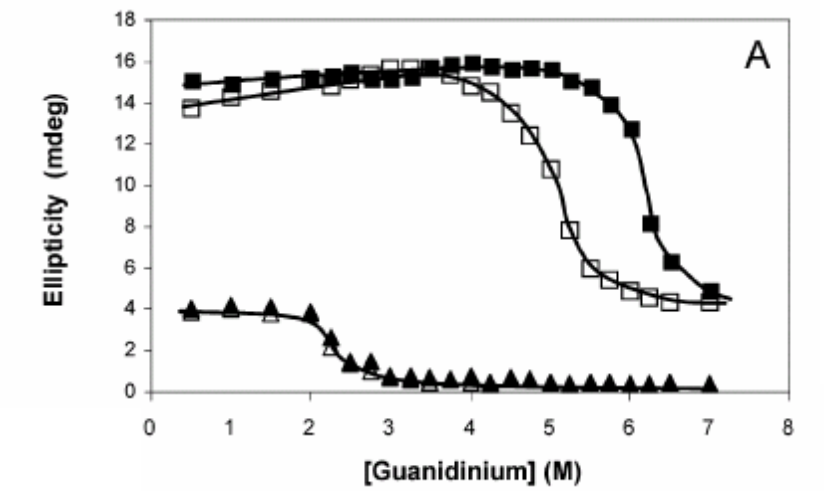

Figure 9*. Guanidinium titration of Ber e 1 at 37 °C*
Native Ber e 1 (squares) and RA-Ber e 1 (triangles) are shown at pH 2.0 (open symbols) and pH 7.0 (closed symbols).

While native Ber e 1 at pH 7.0 is unfolded half-maximally at approximately 6 M guanidinium, approximately 5 M is needed at pH 2.0. RA-Ber e 1, which has already a lower secondary structure content, unfolds at concentrations of guanidinium of 2-3 M, regardless the pH. From these guanidinium tritrations it is concluded that native Ber e 1 is stable more stable at pH 7.0 compared to pH 2.0, at 37 ˚C, and that disulphide bonds are key in maintaining the stabile structure. This is relevant with regard to digestibility as proteins are digested in the stomach at low pH at 37 ˚C. The digestion of native and RA-Ber e 1 was studied in a model for gastric digestion as described by Thomas et al. [6]. Briefly, pepsin was dissolved at pH 2.0 at 37 ˚C and native Ber e 1 or RA-Ber e 1 was added. Samples were collected in time and studied for proteolytic breakdown by SDS-PAGE. LC-MS/MS was used to identify the digestion products. Figure 9 shows the SDS-PAGE analysis.

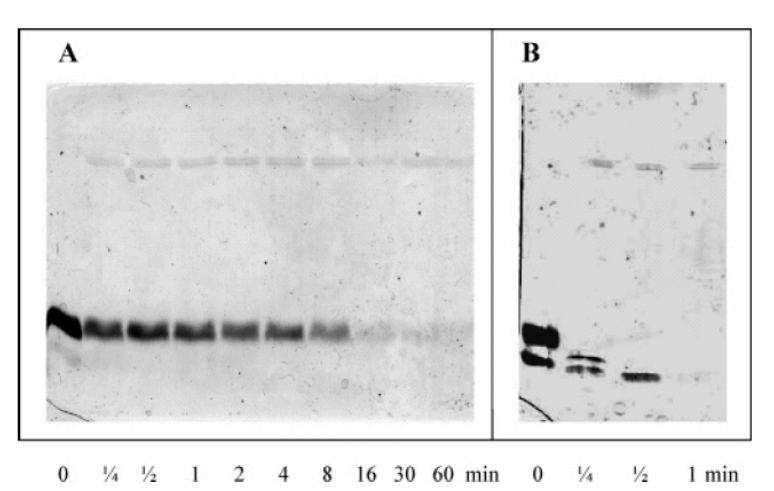

Figure 9. *Pepsin digestion of Ber e 1*
Panel A: Native Ber e 1; Panel B: RA-Ber e 1. X-axes show incubation time in minutes.

While native Ber e 1 remains intact up to 8 minutes, RA-Ber e 1 disappears quickly (< 1 minute). This is in line with the loss to secondary structure and resulting decreased structural stability of RA-Ber e 1 at 37 ˚C (guanidinium titration). LC-MS/MS analysis of the peptides obtained from the digestion of native Ber e 1 indicated that only the heavy chain is affected and only at defined cleavage sites (Figure 10), while for RA-Ber e 1 proteolysis resulted in a large pool of peptides

difficult to analyze. These structure data indicate a loss of protein globularity upon breaking the disulphide bonds, resulting in increased susceptibility for digestion.

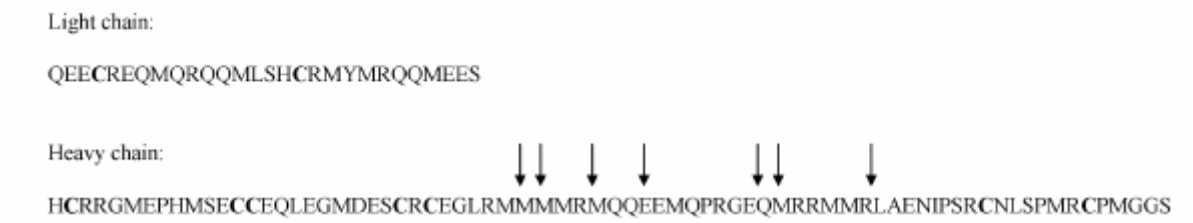

Figure 10. *Sequence of Ber e 1*
Sequence of Ber e 1 according the sequence accession number P04403. Arrows indicate the observed cleavage sites based on mass spectrometric analysis of the peptides obtained after digestion.

3. Conclusions

Several techniques to evaluate the structural stability of a protein are available and by using two examples from earlier work, advantages and disadvantages of these techniques are discussed. Next to these examples some of the techniques have been used to investigate the structural stability of other proteins and allergens. While some of the techniques used for characterizing structural stability are restricted to elevated temperatures, guanidinium-induced protein unfolding and subsequent spectroscopy allows to obtain information of the stability of a protein at for example 37 ˚C, a temperature relevant for digestion.

The structure and structural stability of Ara h 1 from peanuts was assessed by spectroscopic measurements at ambient temperature and elevated temperatures mimicking food processing. Heat treatment of Ara h 1 gives rise to an irreversible endothermic transition of Ara h 1 at 85 ˚C inducing an increase in β-structures, accompanied with aggregation of the protein [3]. Exploring the conditions that provoke such processing-induced aggregation might provide tools to lower the allergenicity.

For Ber e 1, the major allergen from Brazil nut, it was found that heat-treatment would not be an effective way to reduce the allergenicity in view of the thermostabilty of the protein and the complete reversibility of the heat-induced structural changes. Moreover, the stability of the protein structure at 37 ˚C was lower at pH 2.0 compared to pH 7.0, and that the disulphide bonds

were found to be of key importance maintaining the structure. In this case reduction of the disulphide bonds appeared an effective approach to introduce a pronounced loss of secondary structure, providing an increased susceptibility towards proteolysis by pepsin [5].

Concluding, biochemical en biophysical methods to investigate a protein on primary, secondary, tertiary, and quaternary structure are available, and can provide information on the stability of an allergen at ambient as well as elevated temperatures. This information can be used for explaining digestibility behavior of native (raw) allergenic foods and their heat treated (processed) counterparts and may result in a better understanding of protein allergenicity.

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